

# Basic Investigations

## Effects of Suxiao Jiuxin Pill (速效救心丸) on Oxidative Stress and Inflammatory Response in Rats with Experimental Atherosclerosis

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**Objective:** To observe the preventive role of Suxiao Jiuxin Pill (SX 速效救心丸) on atherosclerosis (AS) and to probe into the mechanism in the atherosclerosis rat model.

**Methods:** The AS rat model was established by a high fat diet and a large dose of calcium (vitamin D<sub>3</sub>, 0.6 million U/kg, i.p, once). Sixty healthy male adult Sprague-Dawley (SD) rats were randomly divided into 6 groups, a normal control group (N), a model group (M), a SX low dose group (SXL), a SX middle dose group (SXM), a SX high dose group (SXH), and an atorvastatin group (ATO) ( $n=10$  in each group). The rats in the treatment groups were given with the specific drugs from the first day by oral administration, and the normal control group and the model group were given with normal saline for 12 weeks. Afterwards, the content of malondialdehyde (MDA), the activity of superoxide dismutase (SOD) and the content of oxidized low density lipoprotein (ox-LDL) in the serum were detected. In addition, the expression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) proteins were tested by Western-blot method.

**Results:** The serum ox-LDL and MDA level significantly decreased, SOD activity increased in the SX middle, high dose groups and the atorvastatin group compared to the model group (all  $P<0.05$ ). While the expression of PPAR $\gamma$  and NF- $\kappa$ B proteins significantly decreased in the SX low, middle, high dose groups and the atorvastatin group compared to the model group (all  $P<0.01$ ), with the best effect in the SX high dose group. These results indicate that SX could elevate the activity of serum SOD, decrease serum level of MDA and ox-LDL, and reduce the expression of PPAR $\gamma$  and NF- $\kappa$ B proteins.

**Conclusion:** SX plays an important role in anti-inflammation and inhibition of oxidative stress, which possibly are the mechanism of its preventing and treating atherosclerosis.

**Keywords:** atherosclerosis; rats; Suxiao Jiuxin Pill, oxidized low density lipoprotein; malondialdehyde; superoxide dismutase

The pathogenesis of atherosclerosis (AS) is very complex. A recent study briefly summarized the risk factors of atherosclerosis, namely, hyperlipidemia, hypertension, smoking, diabetes, hyperinsulinemia, genetic factors, gender and age.<sup>1</sup> Oxidative stress and inflammation may be involved in the formation and development of atherosclerosis. When oxidative stress happens, vascular wall cells produce excessive reactive oxygen species (ROS) which can cause peroxidation of lipid and irreversible damage of cell membrane, proteins and DNA. Furthermore, these damages further lead to the oxidative injury of the vascular wall, manifesting as the impairment of the endothelial cell function and structure, migration of monocytes / macrophages, hyperplasia of smooth muscle cells and fibroblasts, and degradation of extracellular matrix. Ultimately, AS develops.<sup>2</sup> Inflammation may promote the formation, development and rupture of atherosclerotic plaque. Previous studies have shown that monocytes / macrophages accumulate in atherosclerotic plaque and produce pro-inflammatory cytokines.<sup>3</sup> Suxiao Jiuxin Pill (SX 速效救心丸) is composed of Chuanxiong (Rhizoma Chuanxiong) and Bingpian (Borneolum) for the treatment of coronary heart disease and angina pectoris. With the character of small doses and rapid effect, SX is widely used as the clinical

emergency medicine with no obvious discomfort, no side effects and no drug resistance.<sup>4</sup> Some studies suggest that SX can promote angiogenesis in the myocardial ischemia area, protecting ischemic myocardium and reducing the size of myocardial infarct.<sup>5</sup> SX can block calcium channel,<sup>6</sup> relax vascular smooth muscle, significantly reduce the blood viscosity, lower the erythrocyte aggregation index and area, and substantially increase the red blood cell deformability.<sup>7</sup> Intensive study also finds that the drug can prevent the formation of AS. In the authors' experiment, they treated experimental atherosclerosis rats by different doses of SX and observed the impact of SX on atherosclerosis and explored its possible mechanisms.

### MATERIALS AND METHODS

#### Animals

Sanitary degree healthy Sprague-Dawley (SD) rats, male, aged 2–3 months old, weighing (256 $\pm$ 20) g, a total of 60, were purchased from the Laboratory Animal Center

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### Experimental Drugs

Vitamin D3 injection was produced by Shanghai General Pharmaceutical Co., 1 mL × 10 pieces/box, batch number: 061001, and used for increasing calcium load so as to establish the atherosclerosis rat model.

SX (40 mg/tablet), a present from the Sixth Chinese Drugs Factor of Tianjin Zhongxin Pharmaceutical Co., was principally composed by Chuan Xiong (Rhizoma Chuanxiong) and Bing Pian (Borneolum).

Atorvastatin, a selective inhibitor of HMG-CoA reductase with the function of reducing plasma cholesterol and lipoprotein levels, was produced by Pfizer Pharmaceuticals, Inc, USA, 20 mg × 7 pieces/box, batch number: 75837003, and used as a control drug.

### Modeling and Grouping

After being fed with ordinary particles food for one week, the animals were randomly divided into 6 groups, normal control group (N), model group (M), SX low dose group (SXL), SX middle dose group (SXM), Suxiao Jiuxin Pill high dose group (SXH), Atorvastatin group (ATO), 10 rats in each group.

The AS rat model was prepared according to the literature.<sup>8</sup> The rats in the model group and the treatment groups were injected intraperitoneally with vitamin D<sub>3</sub>, 0.6 million U/kg, once, and began to feed high fat diet. The rats in the control group were injected with the same volume of saline and fed with basal diet. The experiment lasted 12 weeks.

### Dosages and Methods of Administration

The dosages of adult (SX 15 pills/day, 40 mg/pill; atorvastatin 40 mg/day) were converted into the dosage of the rat according to the weight coefficients: Suxiao Jiuxin Pill low, medium and high dose were 60 mg · kg<sup>-1</sup> · d<sup>-1</sup>, 600 mg · kg<sup>-1</sup> · d<sup>-1</sup>, 1800 mg · kg<sup>-1</sup> · d<sup>-1</sup> respectively, while atorvastatin dosage was 4 mg · kg<sup>-1</sup> · d<sup>-1</sup>. The drugs were grinded into a powder and dissolved in distilled water, and then given by gavage in 0.5 mL/100 g. The drugs were administrated in the treatment groups from the first day of the modeling, while in the model group and the normal control group 0.5 mL/100 g of saline was given, once a day, lasting for 12 weeks. The rats were weighted once a week to adjust the dosages of drugs.

### Specimen Collection

After the last administration, all of the rats were fasted for 12 hours, and then weighed and killed in the next morning. The blood was taken from the femoral artery and centrifuged at 3000 r/min for 15 min. Then the serum

was separated and kept in a refrigerator at -20 °C. The aortic arch was separated and put into a vial and stored in liquid nitrogen immediately for Western blot detection. As some animals died during the experiment, 8 samples were collected randomly in each group.

### Detection of Serum Indicators

Serum content of malondialdehyde (MDA) and serum activity of superoxide dismutase (SOD) were detected by biochemical methods. The content of oxidized low density lipoprotein (ox-LDL) was detected with enzyme-linked immunosorbent assay (ELISA), and the kits used in the experiment were provided by the ADL biotechnology company in USA.

### Detection of Expression of NF-κB and PPARγ Proteins by Western-blot Method

The aortic arch samples were taken from the liquid nitrogen to extract proteins, which were separated by denatured polyacrylamide gel and then the 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, the average luminosity of the gel electrophoresis image was analyzed.

### Data Analysis

SPSS 11.0 software was used for statistical analysis. Measurement data were expressed as mean ± standard deviation ( $\bar{X} \pm s$ ), and the comparison between groups was done by single factor analysis of variance (one-way ANOVA). For pair comparison between groups, LSD method was used when the variance was homogeneous, while DUNNET'S T3 method was used when the variance was arrhythmic.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of SX on ox-LDL Level in AS Rats

As shown in Table 1 and Figure 1, the serum ox-LDL level in the model group was about 5 times higher ( $P < 0.05$ ) than that in the normal control group. Compared to the model group, the serum ox-LDL level in the SX middle, high dose groups and the atorvastatin group significantly decreased ( $P < 0.05$ ), and there was a significant difference in the serum ox-LDL level between the SX middle and high dose groups ( $P < 0.05$ ).

**Table 1.** Serum ox-LDL levels in each group of the rats (μg/dL,  $\bar{X} \pm s$ ,  $n=8$  in each group)

Group	ox-LDL
N	17.26±3.92
M	85.42±6.04 <sup>△</sup>
SXL	85.21±5.52 <sup>▲</sup>
SXM	70.54±4.05 <sup>*</sup>
SXH	53.95±4.64 <sup>*</sup>
ATO	51.97±3.36 <sup>*</sup>

Notes: <sup>△</sup>Compared to the N group,  $P < 0.05$ ; <sup>▲</sup>Compared to M group; <sup>\*</sup>Compared to M group,  $P < 0.05$ .

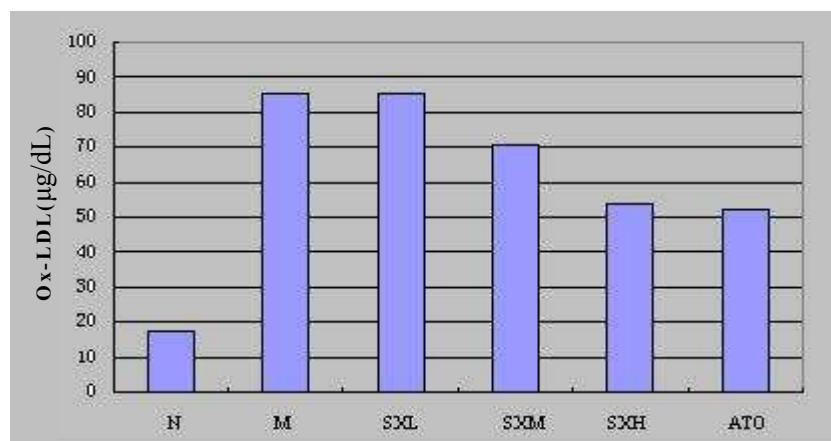


Figure 1. Serum ox-LDL levels in each group of the rats

### Effects of SX on Serum MDA Content and SOD Activity in AS Rats

As seen in Table 2, Figure 2 and Figure 3, the serum MDA content significantly increased and SOD activity decreased in the model group compared to the normal control group (both  $P<0.05$ ). Compared to the model group, serum MDA level decreased and SOD activity increased in the SX middle, high dose groups and the atorvastatin group (all  $P<0.05$ ).

Table 2. Serum MDA contents and SOD activities in each group of the rats ( $\bar{X} \pm s$ ,  $n=8$  in each group)

Group	MDA(nmol/L)	SOD(U/mL)
N	5.17±0.43	165.36±13.80
M	6.44±0.35 <sup>△</sup>	143.05±12.91 <sup>△</sup>
SXL	6.30±0.50	152.41±8.05
SXM	5.73±0.25 <sup>*</sup>	163.46±6.54 <sup>*</sup>
SXH	5.23±0.32 <sup>*</sup>	169.14±4.35 <sup>*</sup>
ATO	5.28±0.44 <sup>*</sup>	166.15±7.80 <sup>*</sup>

Notes: <sup>△</sup>Compared to the N group,  $P<0.05$ ; <sup>\*</sup>Compared to M group,  $P<0.05$ .

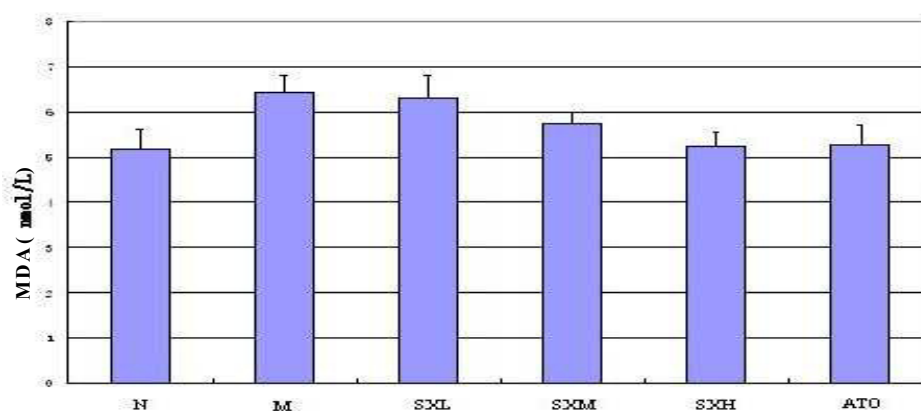


Figure 2. Serum MDA levels in each group of the rat

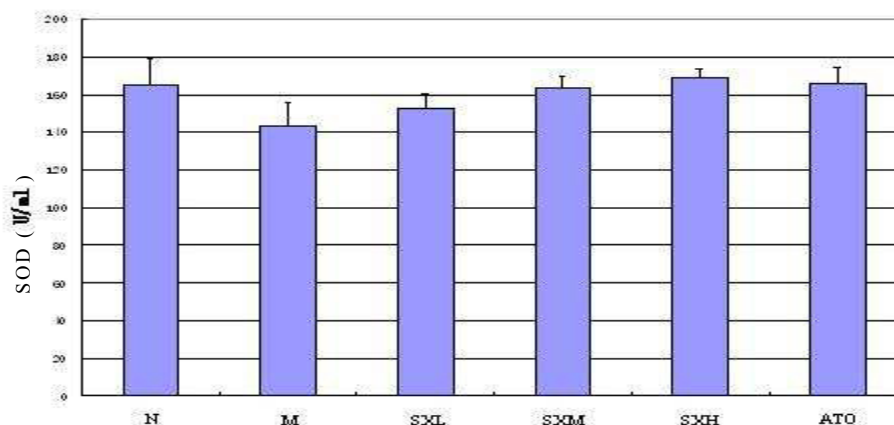


Figure 3. Serum SOD activities in each group of the rats

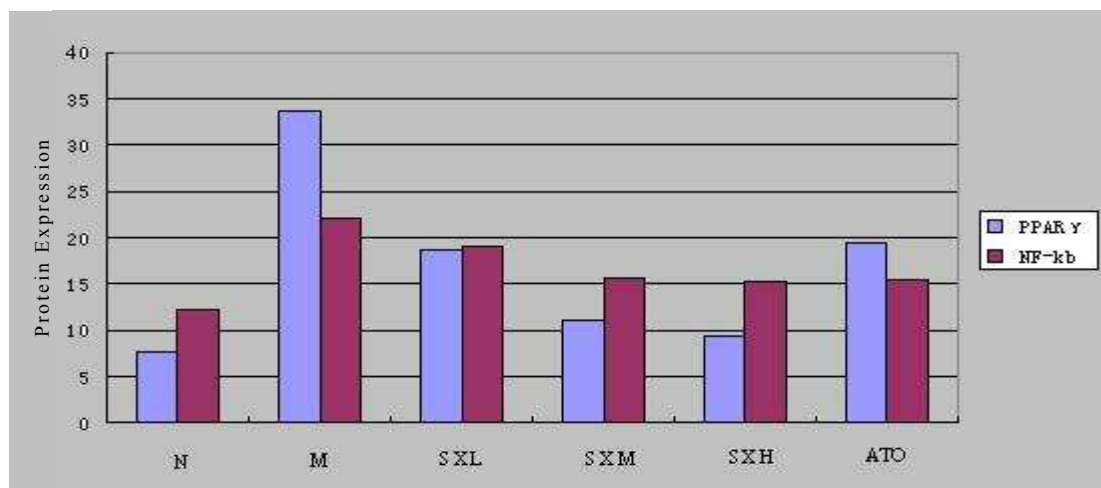
### Expression of PPAR $\gamma$ and NF- $\kappa$ B in the Aortic Arch in the Groups

As shown in Table 3, Figure 4, compared to the normal control group, the expression of PPAR $\gamma$  and NF- $\kappa$ B proteins significantly increased in the model group (both  $P < 0.01$ ); while compared to the model group, the expression of PPAR $\gamma$  and NF- $\kappa$ B proteins significantly decreased in the SX low, middle, high dose groups and the atorvastatin group (all  $P < 0.01$ ), with the best effect in the SX high dose group.

**Table 3.** Expression of PPAR $\gamma$  and NF- $\kappa$ B in the aorta arch in each group of the rats ( $\bar{X} \pm s$ ,  $n=8$  in each group)

Group	PPAR $\gamma$	NF- $\kappa$ B
N	7.59 $\pm$ 0.89	12.20 $\pm$ 0.35
M	33.76 $\pm$ 1.10 <sup>▲</sup>	22.15 $\pm$ 1.07 <sup>▲</sup>
SXL	18.70 $\pm$ 0.56 <sup>*</sup>	19.15 $\pm$ 0.47 <sup>*</sup>
SXM	11.11 $\pm$ 0.94 <sup>*</sup>	15.65 $\pm$ 0.72 <sup>*</sup>
SXH	9.36 $\pm$ 0.92 <sup>▲*</sup>	15.20 $\pm$ 0.89 <sup>*</sup>
ATO	19.48 $\pm$ 0.82 <sup>*</sup>	15.39 $\pm$ 0.60 <sup>*</sup>

Notes: Compared to N group, <sup>▲</sup> $P < 0.01$ ; Compared with M group, <sup>\*</sup> $P < 0.05$ .



**Figure 4.** Expression of PPAR $\gamma$  and NF- $\kappa$ B in the aorta arch in each group of the rats

### DISCUSSION

Atherosclerosis (AS) is a chronic inflammatory lesion in large and middle artery intima.<sup>9</sup> One of the major risk factors causing the chronic inflammatory disease is oxidative modification of lipoproteins, especially, injury of endothelial cells induced by oxidized low density lipoprotein (ox-LDL) and oxidative stress. Antioxidants can reduce or direct quench the free radicals produced by the body, inhibit lipid peroxidation, protect the function of vascular endothelial cells, reduce monocyte adhesion and foam cell formation, and prohibit the proliferation of smooth muscle cells.<sup>10,11</sup>

Studies have shown that by promoting the formation of foam cells, ox-LDL can induce adhesion of monocytes to endothelial cells and chemotaxis of monocytes to subendothelium, promote degradation and proliferation of macrophages, enhance proliferation of endothelial cells and smooth muscle cells, increase adhesion, aggregation and thrombosis of platelet, facilitate vasoconstriction, induce injury of endothelial cells, activate nuclear factor- $\kappa$ B (NF- $\kappa$ B), intensify the inflammatory response of AS, and therefore, promote the initiation and development of AS.<sup>12-15</sup> As a promoter of CD36 receptor,<sup>16</sup> PPAR $\gamma$  can increase the uptake of ox-LDL by macrophage, forming positive feedback, and eventually promote the formation of AS.

It has been proven that NF- $\kappa$ B dimer is inactive in

quiescent cells by binding to its inhibitory protein I $\kappa$ Bs. When it is activated, NF- $\kappa$ B dimer can adjust over 160 genes and their expression products including inflammatory factors and cytokines are involved in the process of atherosclerosis formation.<sup>17,18</sup> Inappropriate activation of NF- $\kappa$ B is the critical step to induce inflammation and oxidative damage, therefore, to control the inappropriate activation of NF- $\kappa$ B is an important strategy for treatment of AS.<sup>19</sup> SOD plays an important role in the balance of oxidation and antioxidant of the body, besides, it can clear the super-anion radicals and protect cells from damage. Thus its activity level reflects the body's ability to eliminate oxygen free radicals. MDA is a stable end product of metabolism after the lipid peroxidation of cell membrane, which can promote cross-linking polymerization of the protein to cause cell injury. In the pathogenesis of AS, the activity of SOD decreases, and the ability to eliminate oxygen free radicals in the body also declines, while MDA regarded as the lipid peroxidized end product increases.

SX is mainly composed of Chuan Xiong (Rhizoma Chuanxiong) and Bing Pian (Borneolum). Chuan Xiong (Rhizoma Chuanxiong) can expand the coronary artery, increase coronary blood flow, reduce the consumption of oxygen in myocardium, and act as the "calcium antagonist".<sup>20</sup> Bing Pian (Borneolum) can extend the time of the body resistance to oxygen, being distributed

rapidly to the heart, lungs, brain and other organs and tissues rich in blood flow.<sup>21</sup>

In the authors' experiment, it was found that the level of ox-LDL and the expression of PPAR $\gamma$  protein in AS rats were significantly higher than those in the normal control group ( $P<0.01$ ), suggesting that ox-LDL and PPAR $\gamma$  play an important role in the occurrence and development of atherosclerosis. These indexes were significantly decreased in the SX middle and high dose groups, indicating that a large number of ox-LDL are generated during the process of AS with activation of the expression of PPAR $\gamma$  protein; SX can inhibit the production of ox-LDL and decrease the expression of PPAR $\gamma$ . It was found that in the SX middle and high dose groups MDA content reduced significantly, meaning that it can repair and protect the integrity of cell membranes. The expression of NF- $\kappa$ B in the aortic arch increased in the model group, while the expression of NF- $\kappa$ B reduced in the SX middle and high dose groups. Intracellular SOD activity in the model group decreased in comparison with that in the normal control group, while the SOD activity in the SX middle and high dose groups were significantly increased as compared to that in the model group, showing that SX can protect SOD activity in cells, thus enhancing antioxidant capacity of aortic cells.<sup>22</sup>

In summary, SX can inhibit oxidative stress and decrease inflammation, so as to play an important role in resisting the occurrence and development of atherosclerosis.

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